POLYCATION-SENSING RECEPTOR IN AQUATIC SPECIES AND METHODS OF USE THEREOF

GOVERNMENT SUPPORT

This invention was made with Government support under Contract No. R01 DK38874 awarded by the National Institutes of Health. The Government has certain rights in the invention.

RELATED APPLICATIONS

This application is a Divisional of U.S. Application No.: 09/715,538, filed on November 17, 2000, entitled, "Polycation-Sensing Receptor in Aquatic Species and Methods of Use Thereof," by H. William Harris, *et al.*, which is a Divisional of U.S. Application No.: 09/162,021, filed on September 28, 1998, entitled, "Polycation-Sensing Receptor in Aquatic Species and Methods of Use Thereof" by H. William Harris, *et al.*, which is a continuation-in-part of International PCT application No. PCT/US97/05031, entitled "Polycation-Sensing Receptor in Aquatic Species and Methods of Use Thereof", by H. William Harris, *et al.*, filed on March 27, 1997, which is a continuation-in-part of Application No. 08/622,738, entitled "Polycation-Sensing Receptor in Fish and Methods of Use Thereof", by H. William Harris, *et al.*, filed March 27, 1996, the teachings of which are hereby incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

It is well recognized that a stagnation or decline in production of edible seafood, in particular, fish, by the marine fishing industry has occurred on a world wide basis. Since the world's population increases by approximately 100 million each year, maintenance of the present caloric content of the average diet will require production of an additional 19 million metric tons of seafood per year (United Nations Food and Agriculture Organization, The State of the World Fisheries and

Aquaculture, Rome, Italy (1995)). In addition, fish products are becoming increasingly utilized in ways other than just food, for example, production of shells and pearls. To achieve this level of production, aquaculture (the cultivation of marine species) will have to double its production in the next 15 years, and wild populations of marine species must be restored.

Aquatic species includes marine teleost and elasmobranch fishes, fresh water teleost fish, euryhaline fish crustations, molusks and echinoderms. Marine teleost fish live in sea water with a high osmolality of about 1,000 mosm. Freshwater teleost fish normally live in water of less than 50 mosm. Euryhaline fish have the ability to acclimate to either of these environments. Ionic composition and osmolality of fish body fluids are maintained in these vastly different environments through gill, kidney and gastrointestinal tract epithelial cell function.

A major problem in aquaculture is development of methodology to rear marine teleost fish, such as cod, flounder and halibut, under freshwater hatchery conditions. To date, factors critical to the acclimation and survival of marine species to fresh water environments, and the control of these factors, have not been fully elucidated.

Attempts to develop such methodologies have also been complicated by problems with feeding the maturing larval forms of these fish. Development of cod, halibut or flounder species that could be reared in fresh water would be of great potential benefit in this regard. Under controlled fresh water conditions, developing forms of these fish could be raised in the absence of bacterial contamination normally present in seawater, and utilize new fresh water food sources that would potentially improve their survival.

The aquaculture industry utilizes the ability of young fish, e.g., salmon, (also called par) to be raised initially in fresh water and subsequently to be transferred for "growth out" in salt water pens as a means to produce large numbers of adult fish (young salmon tolerant to seawater are called smolt). Improvements in both the survival and health of fish undergoing the par-smolt transition would be very valuable for aquaculture growers.

Moreover, salmon that are kept in coastal marine "grow-out" pens during the winter are constantly at risk, since both winter storms, as well as exposure to extremely cold seawater, causes fish to freeze and die. These risks are further complicated by the fact that when adult salmon are adapted to salt water they do not readily readapt back to fresh water environment. Hence, lack of understanding of the means to readapt adult salmon from salt to fresh water results in the loss of salmon.

It is apparent, therefore, that there is an immediate need to develop methods of augmenting the survival of fish in fresh water and sea water, both in a natural environment and an aquacultural environment.

SUMMARY OF THE INVENTION

The present invention relates to the identification and characterization of a PolyValent Cation-sensing Receptor protein (also referred to herein as the Aquatic polyvalent cation-sensing receptor, Aquatic PVCR, or PVCR) which is present in various tissues of marine species. As defined herein, aquatic species includes various fish (e.g., elasmobranch fish, such as sharks, skates; teleost fish, such as summer and winter flounder, salmon, cod, halibut, lumpfish and trout), crustaceans (e.g., lobster, crab and shrimp), mollusks (e.g., clams, mussels and oysters), lamprey and swordfish.

As described herein, for the first time, a polyvalent cation-sensing receptor protein has been identified in aquatic species, located on the plasma membranes of cells in the gastrointestinal tract, kidney, ovary, lung, brain and heart, and in fish brain, gill, heart, intestines, urinary bladder, rectal gland, kidney tubules, and olfactory lamellae. The widespread distribution of Aquatic PVCR protein on the plasma membranes of epithelial cells, as well as in the brain, indicates the involvement of Aquatic PVCR in modulation of epithelial ion and water transport and

endocrine function. Data presented herein demonstrate that the Aquatic PVCR plays a critical role in the acclimation of fish to environments of various salinities. The Aquatic polyvalent cation-sensing receptor allows the successful adaptation of fish, such as flounder, to marine and fresh water environments.

One embodiment of the present invention encompasses Aquatic PVCR proteins expressed in tissues of marine species. Aquatic PVCR proteins have been identified as being present in selected epithelial cells in marine, fresh water and euryhaline fish kidney, intestine, gill, urinary bladder, brain, and olfactory tissue. More specifically, the Aquatic PVCR protein has been identified on the plasma membranes of epithelial cells of fish kidney tubules, especially in the collecting duct (CD), late distal tubule (LDT) and the olfactory lamellae. The present invention is intended to encompass these Aquatic PVCR proteins, their amino acid sequences, and nucleic acid sequences, (DNA or RNA) that encode these Aquatic PVCR proteins. In particular, the claimed invention embodies the amino acid and nucleic acid sequences of PVCRs in dogfish shark, winter and summer flounder, and lumpfish.

In another embodiment of the present invention, methods for regulating salinity tolerance in fish are encompassed. Data presented herein indicate that the Aquatic PVCR is a "master switch" for both endocrine and kidney regulation of adult fish kidney and intestinal ion and water transport, as well as key developmental processes within the fish embryo. Modulating the expression of the Aquatic polyvalent cation-sensing receptor will activate or inhibit Aquatic PVCR mediated ion transport and endocrine changes that permit fish to adapt to fresh or salt water. Also, increasing or deceasing salinity tolerance in aquatic species can refer to activating the PVCR in the epithelial cells.

For example, methods are provided to increase the salinity tolerance of fish adapted to fresh water environment by activation of the Aquatic PVCR in selected epithelial cells. Methods are also provided to decrease the salinity tolerance of fish adapted to a salt water environment by inhibiting the activity of the Aquatic PVCR in selected epithelial cells. Also, regulation of salinity tolerance, via regulating the

activation/inhibition of the Aquatic PVCR, occurs by modulating the ion concentration in the surrounding environment. Such modulation can be done by changing the ion concentration of magnesium, calcium and/or sodium.

In another embodiment of the present invention, methods are provided to identify a substance capable of regulating ionic composition of fish fluids, (e.g., salinity tolerance in fish), and endocrine function, by determining the effect that the substance has on the activation or inhibition of the Aquatic PVCR. As described herein, the nucleic acid sequence encoding an Aquatic PVCR has been determined and recombinant PVCR proteins can be expressed in e.g., oocytes of the frog, Xenopus laevis. The oocyte assay system permits the screening of a large library of compounds that will either activate or inhibit Aquatic PVCR function. Candidate compounds can be further screened in e.g., an in vitro assay system using isolated flounder bladder preparations to measure transepithelial transport of ions important for salinity adaption.

As a result of the work described herein, Aquatic PVCR proteins have been identified and their role in maintaining osmoregulation has been characterized. As a further result of the work described herein, methods are now available to modulate the activation of the Aquatic PVCR, resulting in methods to regulate salinity tolerance in marine and fresh water species of fish and thus, facilitate aquaculture of marine fish. Methods of regulating salinity tolerance also provides the means to develop new species of marine fish that are easily adaptable to fresh water aquaculture. Successful development of new species of marine fish would permit these species to be raised initially in protected fresh water hatcheries and later transferred to marine conditions.

The claimed methods also pertain to method for altering body composition (e.g., tissue composition, or meat/muscle composition) comprising modulating the salinity (e.g., ion concentration) of the surrounding environment. Aspects of body composition that are altered include, but are not limited to: fat content, protein content, weight, thickness, moisture, and taste. For example, the thickness of a filet of fish can be increased by the methods described herein. The altering of body

composition occurs by maintaining the aquatic species in low and/or high salinity/ion concentrations.

The claimed methods also related to methods for reducing or essentially eliminating or ridding the fish of parasites, bacteria, and contaminants. Maintaining aquatic species in higher salinity than normal reduces parasites and/or bacteria while maintaining the species in lower salinity reduces contaminants (e.g., antibiotics, hydrocarbons, and/or amines). The species can be maintained in both environments, consecutively, to reduce parasites, bacteria and contaminants.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-F are photographs of immunocytochemistry results showing the distribution of PVCR protein in various tissues of elasmobranch fish, including dogfish shark (Squalus acanthias) and little skate (Raja crinacca).

Figures 2A-F are photographs of immunocytochemistry results showing the distribution of PVCR protein in various tissues of teleost fish including flounder (Pseudopleuronectes americanus), trout (Onchorynchus nerka) and killifish (Fundulus heteroclitus).

Figures 3A-B are audioradiograms showing RNA blotting analyses.

Figures 4A-E depict the nucleotide sequence of Shark Kidney Calcium Receptor Related Protein-I (SKCaR-RP-I or SKCaR-I) (SEQ ID NO: 1) with the ORF starting at nt 439 and ending at 3516.

Figures 5A-E depict the annotated nucleotide sequence (SEQ ID NO: 1) and the deduced amino acids sequence (SEQ ID NO: 2) of the Shark Kidney Calcium Receptor Related Protein-I (SKCaR-RP-I).

Figure 6 is an autoradiogram showing the results of Northern blot analyses of A+ RNA from various shark tissues.

Figures 7A-B are autoradiograms showing the results of RT-PCR amplifications of poly A+RNA from various aquatic species.

Figure 8 is a photograph of immunocytochemistry results showing PVCR expression in selected tissues of Fundulus after 18 days of exposure to either sea or fresh water as determined by RNA blotting analysis.

Figures 9A-D are photographs showing the results of immunocytochemistry analysis of PVCR expression in the kidney tubules of Fundulus fish either chronically (18 days) or acutely (7 days) adapted to either salt or fresh water.

Figure 10 is a graphical representation showing a normalized calcium response (%) against the amount of Calcium (mM) of the SKCaR-I protein when modulated by alternations in extracellular NaCl concentrations.

Figure 11 is a graphical representation showing a normalized calcium response (%) against the amount of magnesium(mM) of the SKCaR-I protein in increasing amounts of extracellular NaCl concentrations.

Figure 12 is a graphical representation showing the EC50 for calcium activation of shark CaR (mM) against the amount of sodium (mM) of the SKCaR-I protein in increasing amounts of extracellular NaCl concentrations.

Figure 13 is a graphical representation showing the EC50 for magnesium activation of shark CaR (mM) against the amount of sodium (mM) of the SKCaR-I protein in increasing amounts of extracellular NaCl concentrations.

Figure 14 is a graphical representation showing the EC50 for magnesium activation of shark CaR (mM) against the amount of sodium (mM) of the SKCaR-I protein in increasing amounts of extracellular NaCl concentrations and added amounts of calcium (3mM).

Figure 15 is a graphical representation of water transport (Jv) against sequential exposures to $Gd3+$ (300 μM), thiazide (100 μM) and magnesium (100mM) and shows the response of a urinary bladder of winter flounder after exposure of its apical membrane to various CaR agonists and hydrochlorothiazide.

Figure 16A-B are the nucleic acid sequence (cDNA) of a dogfish Shark Calcium Receptor Related Protein-IIa (SKCaR-IIa) (SEQ ID NO: 3).

Figure 17 is the amino acid sequence of a dogfish Shark Calcium Receptor Related Protein-IIa (SKCaR-IIa) (SEQ ID NO:4).

Figure 18A-B is the annotated nucleic (SEQ ID NO: 3) and amino acid sequence (SEQ ID NO:4) for a dogfish Shark Calcium Receptor Related Protein-IIa (SKCaR-IIa).

Figure 19 is the nucleic acid sequence (cDNA) of a dogfish Shark Calcium Receptor Related Protein-IIb (SKCaR-IIb) (SEQ ID NO: 5).

Figure 20 is the amino acid sequence of a dogfish Shark Calcium Receptor Related Protein-IIb (SKCaR-IIb) (SEQ ID NO:6).

Figure 21A-B is the annotated nucleic (SEQ ID NO: 5) and amino acid sequence (SEQ ID NO:6) for a dogfish Shark Calcium Receptor Related Protein-IIb (SKCaR-IIb).

Figure 22 is the nucleic acid sequence (cDNA) of a winter flounder (SEQ ID NO: 7) Aquatic PVCR.

Figure 23 is the amino acid sequence of a winter flounder (SEQ ID NO:8) Aquatic PVCR.

Figure 24A-B is the annotated nucleic (SEQ ID NO: 7) and amino acid sequence (SEQ ID NO:8) for a winter flounder Aquatic PVCR.

Figure 25 is the nucleic acid sequence (cDNA) of a summer flounder (SEQ ID NO: 9) Aquatic PVCR.

Figure 26 is the amino acid sequence of a summer flounder (SEQ ID NO:10) Aquatic PVCR.

Figure 27 is the annotated nucleic (SEQ ID NO: 7) and amino acid sequence (SEQ ID NO:8) for a summer flounder Aquatic PVCR.

Figure 28A-B are the nucleic acid sequence (cDNA) of a lumpfish (SEQ ID NO: 11) Aquatic PVCR.

Figure 29 is the amino acid sequence of a lumpfish (SEQ ID NO:12) Aquatic PVCR.

Figure 30A-C is the annotated nucleic (SEQ ID NO: 11) and amino acid sequence (SEQ ID NO: 12) for lumpfish Aquatic PVCR.

Figure 31 A and B are photographs of immunochemistry of the lamellae of the olfactory organ epithelia of the dogfish shark using antisera 1169 and a control with no antisera 1169, respectively. The darker reaction product indicates specific 1169 antibody binding to the apical membrane of olfactory organ epithelial cells.

DETAILED DESCRIPTION

Described herein, for the first time, are cell surface receptors, called polyvalent cation-sensing receptor proteins, which are present in selected epithelial cells in aquatic species tissue and organs, such as fish kidney, intestine, bladder, rectal gland, gill and brain. This Aquatic receptor protein is also referred to herein as the "Aquatic PVCR" or "PVCR." Evidence is also presented herein that the expression of Aquatic PVCR is modulated in aquatic species transferred from fresh to salt water. The combination of these data and knowledge of osmoregulation in fish, and other marine species, outlined briefly below, strongly suggest that Aquatic PVCR is the "master switch" for both endocrine and kidney regulation of marine species kidney, intestine ion and water transport. In addition, Aquatic PVCR function may control or strongly influence maturation and developmental stages in marine species.

In mammals, calcium receptor protein, or terrestial CaR proteins (also referred to herein as mammalian CaR) have been identified in various tissues in humans and rat. A mammalian CaR protein has been isolated and shown to be the cell surface receptor enabling mammalian parathyroid and calcitonin cells to respond to changes in extracellular Ca^{2+}. (Brown, E.M. *et al.*, New Eng. J. Med., 333:243, (1995)). Mammalian CaR is a membrane protein that is a member of the G-protein-coupled receptor family. When activated by external Ca^{2+}, PVCR modulates various intracellular signal transduction pathways and alters certain functions in selected cells

including secretion of various hormones (PTH, calcitonin, ACTH and prolactin) by endocrine/brain cells and ion transport by epithelial cells.

Subsequent work has revealed that abundant CaR/PVCR is present in epithelial cells of the thick ascending limb (TAL) and distal convoluted tubules (DCT) of the mammalian kidney where it modulates transepithelial salt transport (Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995)). Recent research demonstrated that PVCR is present on the apical surface of epithelial cells of the mammalian kidney medullary collecting duct where it senses urinary Ca^{2+} and adjusts vasopressin-mediated water reabsorption by the kidney (Sands, J.M. *et al.*, J. Clinical Investigation 99:1399-1405 (March 1997)). Lastly, PVCR is also present in various regions of the brain where it is involved in regulation of thirst and associated behavior (Brown, E.M. *et al.*, New England J. of Med., 333:234-240 (1995)).

Another protein important for osmoregulation in mammals is the NaCl cotransporter. The NaCl cotransporter is present in the DCT of human kidney where it absorbs NaCl and facilitates reabsorption of Ca^{2+}. A NaCl cotransporter protein has also been isolated from flounder urinary bladder (Gamba, G. *et al.*, Proc. Nat. Acad. Sci. (USA), 902749-2753 (1993)). Recently, it has been demonstrated that NaCl reabsorption mediated by this NaCl transporter in the DCT of humans is modulated by mammalian PVCR (Plotkin, M. *et al.* J. Am. Soc. Nephrol., 6:349A (1995)).

As described herein, a PVCR protein has been identified in specific epithelial cells in tissues critical for ionic homeostasis in marine species. It is reasonable to believe that the Aquatic PVCR plays similar critical roles in biological functions in marine species, as the mammalian CaR in mammals.

Specifically, Aquatic PVCR proteins have been found in species of elasmobranchs and species of teleosts. Elasmobranchs are cartilaginous fish, such as sharks, rays and skates, and are predominately marine; teleosts, such as summer and winter flounder, cod, trout, killifish and salmon, can be freshwater, marine or euryhaline. The PVCR has also been isolated several other species including lumpfish, swordfish, and lamprey.

Marine teleost fish live in seawater possessing a high osmolality (1,000 mosm) that normally contains 10 millimolar (mM) Ca^{2+}, 50 mM Mg^{2+} and 450 mM NaCl (Evans, D.H. Osmotic and Ionic Regulation, Chapter 11 in The Physiology of Fishes, CRC Press, Boca Raton, FL (1993)). Since their body fluids are 300-400 mosm, these fish are obligated to drink sea water, absorb salts through their intestine and secrete large quantities of NaCl through their gills and Mg^{2+} and Ca^{2+} through their kidneys. Their kidneys produce only small amounts of isotonic urine.

In contrast, fresh water teleost fish possess body fluids of 300 mosm and normally live in water of less than 50 mosm containing 5-20 mM NaCl and less than 1 mM Ca^{2+} and Mg^{2+}. These fish drink little, but absorb large amounts of water from their dilute environment. As a result, their kidneys produce copious dilute urine to maintain water balance. Freshwater fish gill tissue has a low permeability to ions and gill epithelial cells extract NaCl from water (Evans, D.H., "Osmotic and Ionic Regulation", Chapter 11 in The Physiology of Fishes, CRC Press, Boca Raton, FL (1993)).

Euryhaline fish acclimate to various salinities by switching back and forth between these two basic patterns of ion and water transport. For example, when fresh water adapted teleost fish are challenged with high salinities, their gill epithelia rapidly alter net NaCl flux such that NaCl is secreted rather than reabsorbed (Zadunaisky, J.A. *et al.*, Bull. MDI Biol. Lab., 32:152-156 (1992)). Reduction of extracellular Ca^{2+} from 10 mM to 100 micromolar profoundly inhibits this transport process (Zadunaisky, J.A. *et al.*, Bull. MDI Biol. Lab., 32:152-156 (1992)). In flounder species, transfer to seawater activates a series of changes in the kidney allowing for secretion of large quantities of Ca^{2+} and Mg^{2+} by renal epithelia and recovery of water via a thiazide sensitive NaCl cotransporter in the urinary bladder (Gamba, G. *et al.*, Proc. Nat. Acad. Sci. (USA), 90-2749-2753 (1993)).

In a similar fashion, adaption of marine euryhaline fish to fresh water is possible because of a net reversal of epithelial ionic gradients such that NaCl is actively reabsorbed and divalent metal ion secretion ceases (Zadunaisky, J.A. *et al.*,

Bull. MDI Biol. Lab., 32:152-156 (1992)). These changes are mediated by alterations in hormones, especially prolactin, cortisol and arginine vasotocin (Norris, D.O., "Endocrine Regulation of IonoOsmotic Balance in Teleosts", Chapter 16 in Vertebrate Endocrinology, Lea and Febiger, Philadelphia, PA (1985)). These alterations in a cluster of critical hormones and functional changes in epithelial transport in gill, intestine, bladder and kidney are vital not only to rapid euryhaline adaption, but also throughout development of fish embryos, larvae and during metamorphosis.

As described in detail in Example 1, Aquatic PVCR protein has been localized on the plasma membrane of selected epithelial cells in marine species. Specifically, Aquatic PVCR has been located on the apical membrane of epithelial cells of the collecting duct and late distal tubule of the elasmobranch kidney. Aquatic PVCR protein has also been found on the apical membranes of epithelial cells in kidney tubules, gill, urinary bladder and intestine of teleosts. As used herein, the term "apical membrane" or "apical side" refers to the "outside" of the epithelial cell exposed to e.g., urine, rather than the basal side of the cell exposed e.g., to the blood. The apical membrane is also referred to herein as facing the lumen, or interior of e.g., the kidney tubule or intestine. Aquatic PVCR was also found in specific regions of teleost brain.

The Aquatic PVCR has also been localize to the lamellae of the olfactory organ of the dogfish shark. The PVCR was located by using these immunochemistry methods. A detectable antibody, referred herein as antibody/antisera 1169, that is specific to a conserved region of the PVCR was used to find this PVCR. See Figure 31 and Example 8. Aquatic species are able to "smell" or otherwise sense the ion concentrations and/or salinity in their environment.

The Aquatic PVCR proteins, described herein, can be isolated and characterized as to its physical characteristics (e.g., molecular weight, isoelectric point) using laboratory techniques common to protein purification, for example, salting out, immunoprecipation, column chromatography, high pressure liquid

chromatography or electrophoresis. Aquatic PVCR proteins referred to herein as "isolated" are Aquatic PVCR proteins separated away from other proteins and cellular material of their source of origin. These isolated Aquatic PVCR proteins include essentially pure protein, proteins produced by chemical synthesis, by combinations of biological and chemical synthesis and by recombinant methods.

Aquatic PVCR proteins can be further characterized as to its DNA and encoded amino acid sequences as follows: A complementary DNA (cDNA) encoding a highly conserved region of the mammalian CaR, as described in Brown, E.G. *et al.*, Nature, 366:575-580 (1993) or Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995), the teachings of which are incorporated by reference, can be used as a probe to screen a cDNA library prepared from e.g., flounder urinary bladder cells to identify homologous receptor proteins. Techniques for the preparation of a cDNA library are well-known to those of skill in the art. For example, techniques such as those described in Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995), the teachings of which are incorporated herein by reference, can be used. Positive clones can be isolated, subcloned and their sequences determined. Using the sequences of either a full length or several partial cDNAs, the complete nucleotide sequence of the flounder PVCR can be obtained and the encoded amino acid sequence deduced. The sequences of the Aquatic PVCR can be compared to mammalian CaRs to determine differences and similarities.

Similar techniques can be used to identify homologous Aquatic PVCR in other marine species. In particular, a small peptides were used to raise an antibody that is specific to PVCRs. In particular, two antisera were developed. One antisera was raised to a 23-mer peptide, referred as, "4641 antisera or 4641 antibody." A second antisera was raised against a 17-mer peptide, referred to as "1169 antisera" or "1169 antibody." By comparing mammalian receptors and determining a conserved region that is common to all, both the 23-mer and 17-mer peptide were identified and used. The 23-mer peptide has the sequence: DDDYGRPGIEKFREEAEERDICI (SEQ ID

NO.: 13). The 17-mer peptide has the sequence: ARSRNSADGRSGDDLPC.(SEQ ID NO.: 14).

Recombinant Aquatic PVCR proteins can be expressed according to methods well-known to those of skill in the art. For example, PVCR can be expanded in oocytes of the frog, Xenopus laevis, both to prove identity of the cDNA clone and to determine the profile of activation of Aquatic PVCR proteins as compared to mammalian CaR proteins. Exemplary techniques are described in (Brown, E.G. *et al.*, Nature, 366:575-580 (1993); Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995)), the teachings of which are incorporated herein by reference.

As described in Example 2, a 4.4 kb homolog of the mammalian CaR has been found in flounder urinary bladder together with abundant 3.8 kb thiazide-sensitive NaCl cotransporter transcript. Using a homology cloning strategy, a cDNA library from dogfish shark kidney was prepared and screened to obtain multiple cDNA clones with partial homology to mammalian CaRs as described in Example 3. One clone called Shark Kidney-Calcium Receptor Related Protein (SKCaR-RP) was isolated and characterized. SKCAR-RP (also referred to herein as Shark Aquatic PVCR) is 4,131 nucleotides in size (SEQ ID NO: 1). As shown in Figure 4A-F, the complete nucleotide sequence of SKCAR-RP reveals that the clone is composed of 438 nts of 5' untranslated region or UTR followed by a single open reading frame (ORF) of 3,082 nts followed by 610 nts of 3' UTR containing regions of poly A+ RNA. A clone that expresses the shark PVCR was deposited under conditions of the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA on January 28, 1998, under accession number ATCC 209602.

Figure 5A-E show the ORF of the SKCAR-RP in single letter amino acid designations (SEQ ID NO: 2). The deduced amino acid sequence of SKCAR-RP predicts a protein of approximately 110,00 daltons that is 74% homologous to both the rat kidney PVCR protein as well as bovine parathyroid PVCR protein. As described herein, the homology was determined by BLAST software. Analysis of the

amino acid sequence reveals that SKCAR-RP possesses general features that are homologous to PVCR proteins including a large extracellular domain, 7 transmembrane domains and cytoplasmic carboxyl terminal domain. In this regard, many amino acids demonstrated to be critical to PVCR function are identical in SKCAR-RP as compared to mammalian PVCR proteins including specific regions of the extracellular domain and the 7 transmembrane domains. In contrast, other regions are highly divergent, including the amino acids number 351-395 in the extracellular domain as well as the most of the carboxyl terminal region (e.g., amino acids 870-1027). Importantly, the region of amino acids present in mammalian CaRs that was used to generate anti-CaR antiserum is also present in SKCAR-RP.

As shown in Figure 6, Northern blot analysis of mRNA from various shark tissues reveals the highest degree of SKCAR-RP in gill followed by kidney and then rectal gland. These data are highly significant since these tissues have been demonstrated to be involved with ion and water transport and body homeostasis and possess epithelial cells that stain with anti-CaR antiserum. There appears to be at least 3 distinct mRNA species of approximately 7 kb, 4.2 kb and 2.6 kb that hybridize to SKCAR-RP. The 4.2 kb likely corresponds to the SKCAR-RP clone described above.

RT-PCR amplifications were performed as described in Example 3 after isolation of poly A+ RNA from various aquatic species. Primers that permit selective amplification of a region of CaRs (nts 597-981 of RaKCaR cDNA) that is 100% conserved in all mammalian CaRs were utilized to obtain the sequences of similar CaRs in aquatic species. These primers amplify a sequence of 384 nt that is present in the extracellular domain of CaRs and presumably is involved in binding divalent metal ions. The resulting amplified 384 bp cDNA was ligated into a cloning vector and transformed into E. coli cells for growth, purification and sequencing.

As shown in Figures 7A and B, partial cDNA clones have been obtained from: dogfish shark kidney (lane 2), flounder urinary bladder (lane 3), lumpfish liver (lane 5), lobster muscle (lane 8), clam gill (lane 9) and sea cucumber respiratory tissue

(lane 10) using these identical primers. Some tissues (flounder brain-lane 7) did not yield a corresponding 384 nt cDNA despite careful controls. Similarly, no 384 nt cDNA was obtained when only water and not RT reaction mixture was added. These data suggest these 384 nt cDNAs are specific and not expressed in all tissues of aquatic organisms. Each of these 384 nt cDNAs was sequenced and found to contain a conserved nucleotide sequence identical to that present in mammalian CaRs. These data suggest the presence of CaR related proteins in classes of aquatic organisms that are widely divergent in evolution. These include teleost fish (flounder, lumpfish), elasmobranch fish (dogfish shark), crustaceans (lobster), mollusks (clam) and echinoderms (sea cucumber).

It is important to note that Aquatic PVCR sequence obtained from these clones shared complete identity of the 384 nt segment of mammalian CaRs. However, the Aquatic PVCR sequence obtained from the shark kidney clone did not. These data suggest that at least two different classes of aquatic polyvalent cation-sensing receptors exist.

In fact, additional nucleic acid sequences that encodes a PVCR were isolated from the dogfish shark. These nucleic acid sequences, SEQ ID NOs: 3 and 5, are shown in figures 16 and 19, respectively. SEQ ID NO: 3 is 784 nt with an open reading frame coding for 261 amino acids (SEQ ID NO: 4, Figure 17 and 19). SEQ ID NO: 5 is 598 nt long and encodes a 198 amino acid sequence peptide (SEQ ID NO: 6, Figure 20 and 21). It is reasonable to believe that these proteins also sense polyvalent cations, as described herein. The annotated sequences for SEQ ID Nos: 3 and 5 can be found in Figures 18A-B and 21A-B, respectively, along with the deduced amino acid sequences (SEQ ID NOs: 4 and 6). See Example 9.

PVCRs of additional aquatic species have been isolated. For example, nucleic and amino acid sequences for Winter Flounder, Summer Flounder, and Lumpfish have been identified and determined. These sequences were determined using methods described herein and known in the art. The nucleic acid sequences for Winter Flounder (SEQ ID NO: 7), Summer Flounder (SEQ ID NO: 9) and for Lumpfish

(SEQ ID NO: 11) can be found in Figures 22, 25, and 28, respectively. The corresponding deduced amino acid sequences for Winter Flounder (SEQ ID NO: 8), Summer Flounder (SEQ ID NO: 10) and for Lumpfish (SEQ ID NO: 12) can be found in Figures 23, 26, and 29, respectively. See Example 9. Clones, containing sequences for Winter Flounder, Summer Flounder, and Lumpfish were deposited under the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA on October 5, 2000 under accession numbers PTA-2545, PTA-2540, and PTA-2540, respectively.

Additionally, the nucleic and amino acid sequences for an aquatic PVCR were isolated in Swordfish and Lamprey. These sequences were isolated as described herein. These PVCR's function similar to the shark PVCR, as described herein and is capable of sensing ion concentrations/salinity.

The present invention is intended to encompass Aquatic PVCR proteins, and proteins and polypeptides having amino acid sequences analogous to the amino acid sequences of Aquatic PVCR proteins. Such polypeptides are defined herein as Aquatic PVCR analogs (e.g., homologues), or mutants or derivatives. Analogous amino acid sequences are defined herein to mean amino acid sequences with sufficient identity of Aquatic PVCR amino acid sequence to possess the biological activity of an Aquatic PVCR. For example, an analog polypeptide can be produced with "silent" changes in the amino acid sequence wherein one, or more, amino acid residues differ from the amino acid residues of the Aquatic PVCR protein, yet still possesses the biological activity of Aquatic PVCR. Examples of such differences include additions, deletions or substitutions of residues of the amino acid sequence of Aquatic PVCR. Also encompassed by the present invention are analogous polypeptides that exhibit greater, or lesser, biological activity of the Aquatic PVCR proteins of the present invention.

The claimed Aquatic PVCR protein and nucleic acid sequence include homologues, as defined herein. The homologous proteins and nucleic acid sequences can be determined using methods known to those of skill in the art. Initial homology

searches can be performed at NCBI against the GenBank (release 87.0), EMBL (release 39.0), and SwissProt (release 30.0) databases using the BLAST network service. Altshul, SF, et al, Basic Local Alignment Search Tool, J. Mol. Biol. 215: 403 (1990), the teachings of which are incorporated herein by reference. Computer analysis of nucleotide sequences can be performed using the MOTIFS and the FindPatterns subroutines of the Genetics Computing Group (GCG, version 8.0) software. Protein and/or nucleotide comparisons can also be performed according to Higgins and Sharp (Higgins, D.G. and P.M. Sharp, "Description of the method used in CLUSTAL," *Gene, 73*: 237-244 (1988)). Homologous proteins and/or nucleic acid sequences to the PVCR protein and/or nucleic acid sequences that encode the PVCR protein are defined as those molecules with greater than 70% sequences identity and/or similarity (e.g., 75%, 80%, 85%, 90%, or 95% homology).

The "biological activity" of Aquatic PVCR proteins is defined herein to mean the osmoregulatory activity of Aquatic PVCR mammalian PVCR proteins have been shown to mediate physiological responses to changes in body osmolality and salt content in kidney, parathyroid, calcitonin and brain cells. (Brown, E.M. *et al.*, New Eng. J. Med., 333:243, (1995); Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995); Sands, J.M. *et al.*, Nature (Medicine) (1995); Brown, E.M. *et al.*, New England J. of Med., 333:234-240 (1995)). It is reasonable to believe that Aquatic PVCR proteins will possess identical, or similar osmoregulatory activities as these previously identified mammalian CaR proteins in fish kidney, gill, bladder, intestine, rectal gland and brain cells. Assay techniques to evaluate the biological activity of Aquatic PVCR proteins and their analogs are described in Brown, E.M. *et al.*, New Eng. J. Med., 333:243, (1995); Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995); Sands, J.M. *et al.*, Nature (Medicine) (1995); Brown, E.M. *et al.*, New England J. of Med., 333:234-240 (1995), the teachings of which are incorporated herein by reference. Additional assays to evaluate biological activity of PVCR proteins are described in U.S. Serial No. 60/003,697, the teachings of which are also incorporated herein, in its entirety, by reference.

The "biological activity" of Aquatic PVCR is also defined herein to mean the ability of the Aquatic PVCR to modulate signal transduction pathways in specific marine species cells. In mammals, studies in normal tissues, in oocytes using recombinantly expressed CaR, and cultured cells have demonstrated that mammalian CaR protein is capable of complexing with at least two distinct types of GTP-binding (G) proteins that transmit the activation of CaR by an increase in extracellular calcium to various intracellular signal transduction pathways. One pathway consists of mammalian CaR coupling with an inhibitory Gi protein that, in turn, couples with adenylate cyclase to reduce intracellular CAMP concentrations. A second distinct pathway consists of CaR coupling to stimulatory Gq/Gall G protein that couples with phospholipase C to generate inositol 1,4,5 triphosphosphate that, in turn, stimulates both protein kinase C activity and increases intracellular Ca^{2+} concentrations. Thus, depending on the distribution and nature of various signal transduction pathway proteins that are expressed in cells, biologically active mammalian CaRs modulate cellular functions in either an inhibitory or stimulatory manner. It is reasonable to believe that biologically active Aquatic PVCR possesses similar signal transduction activity.

The term "biologically active" also refers to the ability of the PVCR to sense ion concentrations in the surrounding environment. The PVCR senses various polyvalent cations including calcium, magnesium and/or sodium. The PVCR is modulated by varying ion concentrations. For instance, the PVCR may be modulated (e.g., increased expression, decreased expression and/or activation) in response to a change (e.g., increase or decrease) in ion concentration (e.g., calcium, magnesium, or sodium). See Example 6. Responses to changes in ion concentrations of a fish containing a PVCR include the ability for a fish to adapt to the changing ion concentration. Such responses include the amount the fish drinks, the amount of urine output, and the amount of water absorption. Responses also include changes biological processes that affect the body composition of the fish and its ability to excrete contaminants.

The claimed PVCR proteins also encompasses biologically active polypeptide fragments of the Aquatic PVCR proteins, described herein. Such fragments can include only a part of the full-length amino acid sequence of an Aquatic PVCR yet possess osmoregulatory activity. For example, polypeptide fragments comprising deletion mutants of the Aquatic PVCR proteins can be designed and expressed by well-known laboratory methods. Such polypeptide fragments can be evaluated for biological activity, as described herein.

Antibodies can be raised to the Aquatic PVCR proteins and analogs, using techniques known to those of skill in the art. These antibodies polyclonal, monoclonal, chimeric, or fragments thereof, can be used to immunoaffinity purify or identify Aquatic PVCR proteins contained in a mixture of proteins, using techniques well known to those of skill in the art. These antibodies, or antibody fragments, can also be used to detect the presence of Aquatic PVCR proteins and homologs in other tissues using standard immunochemistry methods.

The present invention also encompasses isolated nucleic acid sequences encoding the Aquatic PVCR proteins described herein, and fragments of nucleic acid sequences encoding biologically active PVCR proteins. Fragments of the nucleic acid sequences, described herein, as useful as probes to detect the presence of marine species CaR. Specifically provided for in the present invention are DNA/RNA sequences encoding Aquatic PVCR proteins, the fully complementary strands of these sequences, and allelic variations thereof. Also encompassed by the present invention are nucleic acid sequences, genomic DNA, cDNA, RNA or a combination thereof, which are substantially complementary to the DNA sequences encoding Aquatic PVCR, and which specifically hybridize with the Aquatic PVCR DNA sequences under conditions of stringency known to those of skill in the art, those conditions being sufficient to identify DNA sequences with substantial nucleic acid identity. As defined herein, substantially complementary means that the sequence need not reflect the exact sequence of Aquatic PVCR DNA, but must be sufficiently similar in identity of sequence to hybridize with Aquatic PVCR DNA under stringent conditions.

Conditions of stringency are described in e.g., Ausebel, F.M., *et al.*, Current Protocols in Molecular Biology, (Current Protocols, 1994). For example, non-complementary bases can be interspersed in the sequence, or the sequences can be longer or shorter than Aquatic PVCR DNA, provided that the sequence has a sufficient number of bases complementary to Aquatic PVCR to hybridize therewith. Exemplary hybridization conditions are described herein and in Brown, E.M., *et al.* Nature, 366:575 (1993). For example, conditions such as 1X SSC 0.1% SDS, 50°, or 0.5X SSC, 0.1% SDS, 50° can be used as described in Examples 2 and 3.

The Aquatic PVCR DNA sequence, or a fragment thereof, can be used as a probe to isolate additional Aquatic PVCR homologs. For example, a cDNA or genomic DNA library from the appropriate organism can be screened with labeled Aquatic PVCR DNA to identify homologous genes as described in e.g., Ausebel, F.M., *et al.*, Current Protocols in Molecular Biology, (Current Protocols, 1994).

Typically the nucleic acid probe comprises a nucleic acid sequence (e.g. SEQ ID NO: 1, 3, 5, 7, 9, or 11) and is of sufficient length and complementarity to specifically hybridize to nucleic acid sequences which encode Aquatic species PVCR. The requirements of sufficient length and complementarity can be easily determined by one of skill in the art.

Uses of nucleic acids encoding cloned receptors or receptor fragments include one or more the following: (1) producing receptor proteins which can be used, for example, for structure determination, to assay a molecule's activity on a receptor, and to obtain antibodies binding to the receptor; (2) being sequenced to determine a receptor's nucleotide sequence which can be used, for example, as a basis for comparison with other receptors to determine conserved regions, determine unique nucleotide sequences for normal and altered receptors, and to determine nucleotide sequences to be used as target sites for antisense nucleic acids, ribozymes, hybridization detection probes, or PCR amplification primers; (3) as hybridization detection probes to detect the presence of a native receptor and/or a related receptor in a sample; and (4) as PCR primers to generate particular nucleic acid sequence

regions, for example to generate regions to be probed by hybridization detection probes.

The claimed PVCR proteins and/or nucleic acid sequences include fragment thereof. Preferably, the nucleic acid contains at least 14, at least 20, at least 27, at least 45, and at least 69, contiguous nucleic acids of a sequence provided in SEQ. ID. NO. 1, SEQ. ID. NO. 3, SEQ. ID. NO. 5, SEQ. ID. NO. 7, SEQ. ID. NO. 9, or SEQ. ID. NO. 11. Advantages of longer-length nucleic acid include producing longer-length protein fragments having the sequence of a calcium receptor which can be used, for example, to produce antibodies; increased nucleic acid probe specificity under high stringent hybridization assay conditions; and more specificity for related inorganic ion receptor nucleic acid under lower stringency hybridization assay conditions.

Another aspect of the present invention features a purified nucleic acid encoding an inorganic ion receptor or fragment thereof. The nucleic acid encodes at least 6 contiguous amino acids provided in SEQ. ID. NO. 2, SEQ. ID. NO. 4, SEQ. ID. NO. 6, SEQ. ID. NO. 8, SEQ. ID. NO. 10, SEQ. ID. NO. 12, or SEQ ID NO: 14. Due to the degeneracy of the genetic code, different combinations of nucleotides can code for the same polypeptide. Thus, numerous inorganic ion receptors and receptor fragments having the same amino acid sequences can be encoded for by difference nucleic acid sequences. In preferred embodiments, the nucleic acid encodes at least 12, at least 18, at least 23, or at least 54 contiguous amino acids of SEQ. ID. NO. 2, SEQ. ID. NO. 4, SEQ. ID. NO. 6, SEQ. ID. NO. 8, SEQ. ID. NO. 10, SEQ. ID. NO. 12, or SEQ ID NO: 14.

Another aspect of the present invention features a purified nucleic acid having a nucleic acid sequence region of at least 12 contiguous nucleotides substantially complementary to a sequence region in SEQ. ID. NO. 1, SEQ. ID. NO. 3, SEQ. ID. NO. 5, SEQ. ID. NO. 7, SEQ. ID. NO. 9, or SEQ. ID. NO. 11. By "substantially complementary" is meant that the purified nucleic acid can hybridize to the complementary sequence region in nucleic acid encoded by SEQ. ID. NO. 1, SEQ.

ID. NO. 3, SEQ. ID. NO. 5, SEQ. ID. NO. 7, SEQ. ID. NO. 9, or SEQ. ID. NO. 11 under stringent hybridizing conditions. Such nucleic acid sequences are particularly useful as hybridization conditions, only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having 4 mismatches out of 20 contiguous nucleotides, more preferably 2 mismatches out of 20 contiguous nucleotides, most preferably one mismatch out of 20 contiguous nucleotides. In preferred embodiments, the nucleic acid is substantially complementary to at least 20, at least 27, at least 45, or at least 69 contiguous nucleotides provided in SEQ. ID. NO. 1, SEQ. ID. NO. 3, SEQ. ID. NO. 5, SEQ. ID. NO. 7, SEQ. ID. NO. 9, or SEQ. ID. NO. 11.

Another aspect of the present invention features a purified polypeptide having at least 6 contiguous amino acids of an amino acid sequence provided in SEQ. ID. NO. 2, SEQ. ID. NO. 4, SEQ. ID. NO. 6, SEQ. ID. NO. 8, SEQ. ID. NO. 10, or SEQ. ID. NO. 12. By "purified" in reference to a polypeptide is meant that the polypeptide is in a form (i.e., its association with other molecules) distinct from naturally occurring polypeptide. Preferably, the polypeptide is provided as substantially purified preparation representing at least 75%, more preferably 85%, most preferably 95% or the total protein in the preparation. In preferred embodiments, the purified polypeptide has at least 12, 18, 23, or 54 contiguous amino acids of SEQ. ID. NO. 2, SEQ. ID. NO. 4, SEQ. ID. NO. 6, SEQ. ID. NO. 8, SEQ. ID. NO. 10, or SEQ. ID. NO. 12.

Preferred receptor fragments include those having functional receptor activity, a binding site, epitope for antibody recognition (typically at least six amino acids) (e.g., antisera 1169). Such receptor fragments have various uses such as being used to obtain antibodies to a particular region and being used to form chimeric receptors with fragments of other receptors create a new receptor having unique properties.

The invention also features derivatives of full-length inorganic ion receptors and fragments thereof having the same, or substantially the same, activity as the full-length receptor or fragment. Such derivatives include amino acid addition(s),

substitution(s), and deletion(s) to the receptor which do not prevent the derivative receptor from carrying out one or more of the activities of the parent receptor.

Another aspect of the present invention features a recombinant cell or tissue. The recombinant cell or tissue is made up of a recombined nucleic acid sequence encoding at least 6 contiguous amino acids provided in SEQ. ID. NO. 2, SEQ. ID. NO. 4, SEQ. ID. NO. 6, SEQ. ID. NO. 8, SEQ. ID. NO. 10, or SEQ. ID. NO. 12 and a cell able to express the nucleic acid. Recombinant cells have various uses including acting as biological factories to produce polypeptides encoded for by the recombinant nucleic acid, and for producing cells containing a functioning PVCR protein. Cells containing a functioning PVCR can be used, for example, to screen to antagonists or agonists.

As described in Example 4, it is demonstrated that the Aquatic PVCR protein plays a critical role in the adaption of euryhaline fish to environments of various salinities. Adaption of the killifish, *Fundulus heteroculitus*, to seawater resulted in steady state expression of Aquatic PVCR MRNA in various tissues.

It is also demonstrated herein that PVCR protein undergoes rearrangement within epithelial cells of the urinary bladder in flounder adapted to brackish water as compared to full strength sea water. This directly correlates with alterations the rate of NaCl transport by these cells.

Preliminary experiments shows that winter flounder were adapted to live in 1/10th seawater (100 mOsm/kg) by reduction in salinity from 450 mM NaCl to 45 mM NaCl over an interval of 8 hrs. (Further experimentation illustrated that winter and summer flounder can be maintained in 1/10 or twice the salinity for over a period of 6 months.) After a 10 day interval where these fish were fed a normal diet, the distribution of the PVCR in their urinary bladder epithelial cells was examined using immunocytochemistry. PVCR immunostaining is reduced and localized primarily to the apical membrane of epithelial cells in the urinary bladder. In contrast, the distribution of PVCR in epithelial cells lining the urinary bladders of control flounders continuously exposed to full strength seawater is more abundant and present in both

the apical membranes as well as in punctate regions throughout the cell. These data are consistent with previous Northern data since more PVCR protein is present in the urinary bladders of seawater fish vs fish adapted to brackish water. These data suggest that PVCR protein may be present in vesicles in epithelial cells of the urinary bladder and that in response to alterations in salinity, these vesicles move from the cell cytoplasm to the apical surface of these epithelial cells. Since these same epithelial cells possess abundant NaCl cotransporter protein that is responsible for water reabsorption in the urinary bladder, these data suggest that the PVCR protein modulates NaCl transport in the flounder urinary bladder by altering the proportion of NaCl cotransporter protein that is present in the apical membrane. As urinary Mg^{2+} and Ca^{2+} concentrations increase when fish are present in full strength sea water, activation of apical PVCR protein causes endocytosis and removal of NaCl cotransporter from the apical membrane and thus reduction in urinary bladder water transport.

As a result of the work described herein, methods are now provided that facilitate euryhaline adaptation of fish to occur, and improve the adaption. More specifically, methods are now available to regulate salinity tolerance in fish by modulating (e.g., alternating, activating and or expressing) the activity of the Aquatic PVCR protein present in epithelial cells involved in ion transport, as well as in endocrine and nervous tissue. For example, salinity tolerance of fish adapted (or acclimated) to fresh water can be increased by activating the Aquatic PVCR, for example, by increasing the expression of Aquatic PVCR in selected epithelial cells, resulting in the secretion of ions and seawater adaption. Specifically, this would involve regulatory events controlling the conversion of epithelial cells of the gill, intestine and kidney. In the kidney, PVCR activation will facilitate excretion of divalent metal ions including Ca^{2+} and Mg^{2+} by renal tubules. In the gill, PVCR activation will reduce reabsorption of ions by gill cells that occurs in fresh water and promote the net excretion of ions by gill epithelia that occurs in salt water. In the

intestine, PVCR activation will permit reabsorption of water and ions across the G.I. tract after their ingestion by fish.

Alternatively, the salinity tolerance of fish adapted to seawater can be decreased by inhibiting the Aquatic PVCR, for example, by decreasing the expression of Aquatic PVCR in selected epithelial cells, resulting in alterations in the absorption of ions and freshwater adaption. Selected epithelial cells include, e.g., kidney, bladder, intestinal and gill cells.

The presence of Aquatic PVCR in brain reflects both its involvement in basic neurotransmitter release via synaptic vesicles (Brown, E.M. *et al.*, New England J. of Med., 333:234-240 (1995)), as well as its activity to trigger various hormonal and behavioral changes that are necessary for adaptation to either fresh water or marine environments. For example, increases in water ingestion by fish upon exposure to salt water is mediated by PVCR activation in a manner similar to that described for humans where PVCR activation by hypercalcemia in the subfornical organ of the brain cause an increase in water drinking behavior (Brown, E.M. *et al.*, New England J. of Med., 333:234-240 (1995)). In fish, processes involving both alterations in serum hormonal levels and behavioral changes are mediated by the brain. These include the reproductive and spawning of euryhaline fish in fresh water after their migration from salt water as well as detection of salinity of their environment for purposes of feeding, nesting, migration and spawning.

Data obtained recently from mammals now suggest that PVCR activation plays a pivotal role in coordinating these events. For example, alterations in plasma cortisol have been demonstrated to be critical for changes in ion transport necessary for adaptation of salmon smolts from fresh water to salt water (Veillette, P.A., *et al.*, Gen. and Comp. Physiol., 97:250-258 (1995). As demonstrated recently in humans, plasma Adrenocorticotrophic Hormone (ACTH) levels that regulate plasma cortisol levels are altered by PVCR activation.

"Salinity" refers to the concentration of various ions in a surrounding aquatic environment. In particular, salinity refers to the ionic concentration of calcium,

magnesium and/or sodium (e.g., sodium chloride). "Normal salinity" levels refers to the range of ionic concentrations of typical water environment in which an aquatic species naturally lives. For winter and summer flounder, normal salinity or normal seawater concentrations are about 10mM Ca, 25mM Mg, and 450 mM NaCl. "Salinity tolerance" refers to the ability of a fish to live or survive in a salinity environment that is different than the salinity of its natural environment. The upper or lower limit of ionic concentrations in which the fish can survive have been defined. Salinity tolerance of a fish has been defined to be between at least 4X and 1/50, or 3X and 1/25, or preferably, twice and 1/10 the normal salinity.

Winter and summer flounder were maintained in at least twice the normal salinity or 1/10 the normal salinity. See Example 10. These fish can be maintained in these environments for long periods of time (e.g., over 3 months, over 6 months, or over 1 year). These limits were defined by decreasing or increasing the ionic concentrations of calcium, magnesium, and sodium, keeping a constant ratio between the ions. These salinity limits can be further defined by increasing and/or decreasing an individual ion concentration, thereby changing the ionic concentration ratio among the ions. Increasing and/or decreasing individual ion concentrations can increase and/or decrease salinity tolerance. "Hypersalinity" or "above normal salinity" levels refers to a level of at least one ion concentration that is above the level found in normal salinity. "Hyposalinity" or "below normal salinity" levels refers to a level of at least one ion concentration that is below the level found in normal salinity.

Maintaining winter and summer founder in this environment for about 3 months induced noticeable and significant changes occurred to the body composition of the flounder. These fish were slowly adapted to the hypersalinity or hyposalinity environments over a period of 15 days. Body composition refers to various characteristics of the fish, including, but not limited to, weight, muscle, fat, protein, moisture, taste, or thickness. Alteration of the body composition means inducing a change in one of these characteristics. Maintaining fish in 1/10 the normal salinity results in a fish that is twice as thick, 70% fatter, and "less fishy," (e.g., milder

flavor) tasting fish than those fish maintained in hypersalinity environments. See Example 10. A fish maintained in low salinity or hyposalinity can increase its fat content by at least 10% or 20%, and preferably by at least 30%, 40%, or 50% than those fish maintained in normal salinity. Similarly, a fish maintained in low salinity or hyposalinity can increase its thickness by at least 30% or 40%, and preferably by at least 50%, 60%, or 70% than those fish maintained in normal salinity. A fish maintained in high salinity or hypersalinity can decrease its fat content by at least 10% or 20%, and preferably by at least 30%, 40%, or 50% than those fish maintained in normal salinity. Similarly, a fish maintained in high salinity or hypersalinity can decrease its thickness by at least 30% or 40%, and preferably by at least 50%, 60%, or 70% than those fish maintained in normal salinity.

Maintaining fish in a hypersalinity environment also results in fish with a reduced number of parasites or bacteria. Preferably, the parasites and/or bacteria are reduced to a level that is safe for human consumption, raw or cooked. More preferably, the parasites and/or bacteria are reduced to having essentially no parasites and few bacteria. These fish must be maintained in a hypersalinity environment long enough to rid the fish of these parasites or bacteria, (e.g., for at least a few days or at least a few weeks).

The host range of many parasites is limited by exposure to water salinity. For example, *Diphyllobothrium* species commonly known as fish tapeworms, is encountered in the flesh of fish, primarily fresh water or euryhaline species including flounder of salmon. Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. 1991. US Food and Drug Administration Center for Food Safety and Applied Nutrition, the teachings of which are incorporated herein by reference in their entirety. In contrast, its presence in the flesh of completely marine species is much reduced or absent. Since summer flounder can survive and thrive at salinity extremes as high as 58 ppt (1.8 times normal seawater) for extended periods in recycling water, exposure of summer flounder to hypersalinity conditions might be used as a

"biological" remediation process to ensure that no *Diphyllobothrium* species are present in the GI tract of summer flounder prior to their sale as product.

Recent data from Cole et al, J. Biol. Chem. 272:12008-12013, 1997, (the teachings of which are incorporated herein by reference in their entirety) show that winter flounder elaborate an antimicrobial peptide from their skin to prevent bacterial infections. Their data reveals that in the absence of pleurocidin, *E. coli* are killed by high concentrations of NaCl. In contrast, low concentrations of NaCl(< 300mM NaCl) allow *E. Coli* to grow and under these conditions pleurocidin presumably helps to kill them. These data provide evidence of NaCl killing of *E. Coli*, as well as highlight possible utility of bacterial elimination in fish.

Similarly, maintaining fish in a hyposalinity environment results in a fish with a reduced amount of contaminants (e.g., hydrocarbons, amines or antibiotics). Preferably, the contaminants are reduced to a level that is safe for human consumption, raw or cooked, and produces a milder, "less fishy" tasting fish. More preferable, the contaminants are reduced to having essentially very little contaminants left in the fish. These fish must be maintained in a hyposalinity environment long enough to rid the fish of these contaminants, (e.g., for at least a few days or a few weeks).

Organic amines, such as trimethylamine oxide (TMAO) produce a "fishy" taste in seafood. They are excreted via the kidney in flounder. (Krogh, A. Osmotic Regulation in Aquatic Animals, Cambridge University Press, Cambridge, U.K. pgs 1-233, 1939, the teachings of which are incorporated herein by reference in their entirety). TMAO is synthesized by marine organisms consumed by fish that accumulate the TMAO inn their tissues. Depending on the species of fish, the muscle content of TMAO and organic amines is either large accounting for the "strong" taste of bluefish and herring or small such as in milder tasting flounder.

TMAO is an intracellular osmolyte and its accumulation in cells prevents osmotic loss of water produces by hypertonic seawater (Forster, RP and L Goldstein, Formation of Excretory Products Chapter 5 in Fish Physiology, Edited by WS Hoar,

DJ Randall and JR Brett Volume VIII Bioenergics and Growth. Academic Press, New York, NY pages 313-345, 1969, the teachings of which are incorporated herein by reference in their entirety). The excretion of TMAO by marine teleost fish such as winter flounder occurs almost exclusively via the kidney. Thus, in low salinities urine flow in winter flounder is high and dietary amines including TMAO are almost completely excreted. Elger, E. B. Elger, H. Hentschel and H. Stolte, Adaptation of renal function to hypotonic medium in the winter flounder (*Psuedupleuronecies americanus*). J. Comp. Physio. B157:21-30 (1987), the teachings of which are incorporated herein in their entirety. In full strength or hyperosmotic seawater, urine flow is much diminished and amine excretion is greatly reduced and therefore accumulates in the flounder muscle. Thus, muscle levels of amines can be altered by subjecting flounder to differing osmotic environments and likely result in winter flounders with differing tastes.

Exposure of either winter or summer flounders to waters of extreme differences in salinity (3-4 vs 58 ppt) produces profound changes in the kidney function of these fish that allow toxic compounds such as antibiotics and heavy metal to be excreted. At low salinities (3-4 ppt) the glomerular filtration and urinary flow rates are 10-100 fold larger as compared to identical fish exposed to full strength seawater. High glomerular filtration and urine flow rates provide for a large increase in the clearance of a variety of organic compounds including antibiotics used in aquaculture (Physicians Desk Reference, 49th Edition, Medical Economics Data Production Company, Montvale, NY page 2103, the teachings of which are incorporated herein by reference in their entirety), as well as heavy metals including Ni^{2+}, Pb^{2+} (Forster, RP and L Goldstin. 1969. Formation of Excretory Products Chapter 5 in Fish Physiology, Edited by WS Hoar, DJ Randall and JR Brett Volume VIII Bioenergics and Growth. Academic Press. New Your, NY pages 313-345 (the teachings of which are incorporated herein by reference in their entirety)). Exposure of flounder to an interval of low salinity prior to market would produce high urine flow rates and, therefore, reduce any tissue burdens of toxic or antibiotic compounds

acquired during growth. This method serves as a effective strategy to reduce environmental contaminants to their lowest levels possible.

Methods encompassed by the present invention include methods of activating or deactivating the Aquatic PVCRs described herein. The term "activation" as used herein means to make biologically functional, e.g., rendering a cell surface receptor capable of stimulating a second messenger which results in modulation of ion secretion. This could be in the form of either an inhibition of signal transduction pathways, e.g., via a Gi protein, or stimulation of other pathways via. e.g., a Gq/Gall protein. As a result of these alterations, ion transport by epithelial cells is reduced or stimulated. Also, activation can be related to expression (e.g., an increase in expression).

For example, a compound, or substance, which acts as an agonist can interact with, or bind to, the Aquatic PVCR, thereby activating the Aquatic PVCR, resulting in an increase of ion secretion in selected epithelial cells. An agonist can be any substance, or compound, that interacts with, or binds to, the Aquatic PVCR resulting in activation of Aquatic PVCR. Agonists encompassed by the present invention include inorganic ions, such as the polyvalent cations calcium, magnesium and gadolinium, and organic molecules such as neomycin. Other agonists, include inorganic compounds, nucleic acids or proteins can be determined using the techniques described herein.

Agonists also encompassed by the present invention can include proteins or peptides or antibodies that bind to the Aquatic PVCR resulting in its activation. Activation of the Aquatic PVCR is typically direct activation. For example, an inorganic molecule or peptide binds directly to the receptor protein resulting in the activation of Aquatic PVCR. However, activation of the Aquatic PVCR can also be indirect activation, such as would occur when e.g., an antibody is available to bind an Aquatic PVCR antagonist, thus permitting activation of the Aquatic PVCR

The term "deactivation" or "inactivation" as used herein means to completely inhibit or decrease biological function. For example, deactivation is when a cell

surface receptor is incapable of stimulating a second messenger. Specifically, as used herein, deactivation of the Aquatic PVCR occurs when the Aquatic PVCR is rendered incapable of coupling with, or stimulating, a second messenger, resulting in the absorption of ions in selected epithelial cells. Deactivation can be direct or indirect. For example, an antagonist can interact with, or bind directly to the Aquatic PVCR, thereby rendering the Aquatic PVCR incapable of stimulation of a messenger protein.

Alternatively, deactivation can be indirect. For example, an antagonist can deactivate Aquatic PVCR by preventing, or inhibiting an agonist from interacting with the Aquatic PVCR. For example, a chelator can bind calcium ions and, thus prevent the calcium ions from binding to the Aquatic PVCR. Antagonists of the Aquatic PVCR can be any substance capable of directly interacting with, or binding to, the Aquatic PVCR or interacting with, or binding to, an agonist of the Aquatic PVCR that results in deactivation of the Aquatic PVCR. Antagonists encompassed by the present invention can include, for example, inorganic molecules, organic molecules, proteins or peptides. Antagonists can also be nucleic acids, such as anti-sense DNA or RNA sequences that bind to the DNA encoding the Aquatic PVCR, thereby preventing or inhibiting transcription into MRNA. Antagonists can also be anti-sense RNA that binds to the PVCR transcript, thereby preventing, or inhibiting translation.

Candidate substances, (e.g., compounds, peptIdes or nucleic acids) to be evaluated for their ability to regulate Aquatic PVCR activity can be screened in assay systems to determine activity. For example, one assay system that can be used is the frog oocyte system expressing Aquatic PVCR described in Brown, E.G. *et al.*, Nature, 366:575-580 (1993); Riccardi, D.J. *et al.*, Proc. Nat. Acad. Sci USA, 92:131-135 (1995).

A functional assay to screen for compounds that alter PVCR mediated NaCl transport function in adult flounder urinary bladder can also be used to screen candidate compounds for their ability to modulate Aquatic PVCR. Transport of NaCl via the thiazide sensitive NaCl cotransporter in the flounder urinary bladder is important in its adaptation to various salinities. NaCl transport is readily quantified

using a isolated bladder preparation from adult flounder and measurement of transepithelial Ca^{2+} sensitive short circuit current, as described in (Gamba, G. *et al.*, Proc. Nat. Acad. Sci. (USA), 90-2749-2753 (1993)). Use of this isolated in vitro assay system can establish a direct effect of Aquatic PVCR function or transepithelial transport of ions important for salinity adaptation. Compounds identified using the frog oocyte assay and in vitro NaCl transport assay system can be further tested in whole animal adaptation experiments.

For example, to screen for PVCR reactive compounds (both agonists and antagonists) an assay previously used for study of ion and water transport in isolated flounder urinary bladders (Renfro, L.J. Am. J. Physiol. 228:52-61, 1975) has been used. As described herein (Example 5), this assay has now been adapted to screen PVCR agonists and provided data showing that water reabsorption is >85% inhibited by application of thiazide (specific inhibitor of the thiazide sensitive NaCl cotransporter); water reabsorption is >90% inhibited by application of gadolinium (a PVCR specific agonist); water reabsorption is >50% inhibited by application of neomycin (a PVCR specific agonist); and exposure of the bladder to PVCR agonists is reversible upon removal of either gadolinium or neomycin.

As a further result of the work, methods are provided to test the function of PVCR in developing fish, and to specifically select for fish with altered PVCR functional and osmotic tolerance. The developmental expression of PVCR in developing embryo, larval and metamorphic forms of fish can be determined using antibodies that recognize Aquatic PVCR and/or mammalian CaR, or by using Aquatic and/or mammalian cDNA probes, or a combination of these techniques. Initial screening of gametes, larval or metamorphic forms of fish can be tested using immunohistochemistry, such as described in Example 1, to determine at what stage of development the PVCR protein is expressed in developing fish.

Based on the immunochemistry studies of the Aquatic PVCR structure, function and developmental expression, specific selection assays can be designed to identify fish, e.g., flounder, halibut or cod, species with altered Aquatic PVCR

function that can survive in fresh water, while those possessing normal PVCR function will die. These acute survival assays can evaluate the overall effect of PVCR agonists and antagonists identified by e.g., the frog oocyte expression assay. These assays will test the potency of various PVCR active compounds on improving or reducing survival of various fish or embryos. The ability to identify a single individual fish with alterations in PVCR function and osmoregulation from many wild type fish possessing normal characteristics will permit the propagation of specific strains of fish that exhibit specific salinity tolerance characteristics. Development of larval forms of cod, halibut or flounder that survive in fresh water can then be utilized in experiments to test whether new food sources could be used in their rearing. Successful development of these goals would then permit these species to be raised initially in protected fresh water hatcheries and later transferred to marine conditions similar to those presently utilized for aquaculture of salmon.

Also encompassed by the present invention are methods of modulating the activation of the Aquatic PVCR by altering the DNA encoding the Aquatic PVCR, and thus, altering the subsequent expression of Aquatic PVCR protein in various tissues. For example, anti-sense nucleic acid sequences (either DNA or RNA) can be introduced into e.g., epithelial cells in fish kidney, where the anti-sense sequence binds to the Aquatic PVCR gene and inhibits, or substantially decreases its transcription into MRNA. Alternatively, the anti-sense sequence can bind to the Aquatic PVCR MRNA and inhibit, or substantially decrease, its translation into amino acid sequence.

Alternatively, a mutated or chimeric Aquatic PVCR gene construct (e.g., a mutated or chimeric SEQ ID NO: 1) can be inserted into, e.g. fish eggs, to produce new marine strains with enhanced, or decreased, Aquatic PVCR protein activity. The anti-sense sequence or gene construct is introduced into the cells using techniques well-known to those of skill in the art. Such techniques are described in Hew, C.L., *et al.*, Mol. Aquatic Biol. Biotech., 1:380717 (1992) and Du, S.J., *et al.*,

Biotechnology, 10:176-181 (1992), the teachings of which are incorporated herein by reference.

Based on the work described herein, new methodologies that will regulate the adaptation of fish, particularly flounder, halibut and cod, to environments of varying salinities are now available. For example, methods are now available to adapt developing forms of flounder, halibut or cod to fresh water environments. Rearing of these species in fresh water will allow for new approaches to the problems of feeding and successful rearing of larval forms of these fish species. Methods are also now available for selection and propagation of new strains of fish (e.g., flounder, halibut and cod) that will possess alterations in their salinity tolerance such that they can be raised in fresh water, then transferred to seawater. This approach has many advantages since it will both diversify the aquaculture industry and make use of existing hatcheries and facilities to produce flounder, cod or halibut as well as salmon.

The present invention is illustrated by the following Examples, which are not intended to be limited in any way.

EXAMPLE 1: IMMUNOHISTOCHEMISTRY OF THE PVCR PROTEIN PRESENT IN AQUATIC SPECIES EPITHELIAL CELLS

Tissues from fish were fixed by perfusion with 2% paraformaldehyde in appropriate Ringers solution corresponding to the osmolality of the fish after anesthesitizing the animal with MS-222. Samples of tissues were then obtained by dissection, fixed by immersion in 2% paraformaldehyde, washing in Ringers then frozen in an embedding compound, e.g., O.C.T.™ Miles, Inc. Elkahart, Indiana, using methylbutane cooled with liquid nitrogen. After cutting 4μM tissue sections with a cryostat, individual sections were subjected to various staining protocols. Briefly, sections mounted on glass slides were: 1) blocked with serum obtained from the species of fish, 2) incubated with rabbit anti-CaR antiserum and 3) washed and incubated with peroxidase conjugated affinity purified goat antirabbit antiserum. The locations of the bound peroxidase conjugated goat antirabbit antiserum was visualized

by development of a rose colored aminoethylcarbazole reaction product. Individual sections were mounted, viewed and photographed by standard light microscopy techniques. The anti-CaR antiserum used to detect fish PVCR protein was raised in rabbits using a 23-mer peptide corresponding to amino acids numbers 214-236 localized in the extracellular domain of the RaKCaR protein.

In both species of elasmobranchs studied, (dogfish shark, *Squatus Acanthias* and little skate, *Raja Erinacea*), PVCR protein was localized to the apical membranes of selected epithelial cells. The distribution of PVCR in elasmobranch tissue is shown in Figures 1A-F. Heavy black coloring is displayed where anti-CaR antibody binding is present consistently in areas of tissues designated by arrowheads. Figure 1A: Kidney-CaR expression is present on apical membranes of epithelial cells of late distal tubule (LDT) and collecting duct (CD). Figure 1B: Gill PVCR expression is localized to epithelial cells of gill arcades. Figure 1C: Brain PVCR expression is localized to distinct groups of neurons in the brain. Figure 1D: Rectal gland PVCR expression is localized to apical membranes of cells lining the ducts of the rectal gland. Figure 1E: Intestine PVCR expression is localized to the apical membranes of epithelial cells lining the lumens of the intestine. Figure 1F: Ovary PVCR expression is present in both oocytes and surrounding follicular cells.

Figures 2A-F show the distribution of PVCR in the flounder (*Pseudopleuronectes americanus*) and in the fresh water trout (*Onchorynchus Nerka*). Figures 2A-F display heavy black coloring where anti-CaR antibody binding is present consistently in areas of tissues designated by arrowheads. Figure 2A: Kidney-CaR expression is present on apical membranes of epithelial cells of large tubules (LT) and collecting ducts (CD). Figure 2B: Gill PVCR expression is localized to epithelial cells of gill arcades. Figure 2C: Brain PVCR expression is localized to distinct groups of neurons in the brain. Figure 2D: Urinary bladder PVCR expression is localized to apical membranes of cells lining the urinary bladder. Figure 2E: Intestine PVCR expression is localized to the apical membranes of epithelial cells

lining the lumens of the intestine. Figure 2F: Ovary PVCR expression is present in both oocytes and surrounding follicular cells.

EXAMPLE 2: RNA BLOTTING ANALYSES OF WINTER FLOUNDER TISSUE

Five microgram samples of poly A+ RNA prepared from various winter flounder tissues including muscle (lane 1), heart (lane 2), testis (lane 3) and urinary bladder (lane 4) were subjected to RNA blotting analyses (Figures 3A and B).

As shown in Figure 3A, a single filter was first hybridized using a ^{32}P-labeled ECO Rl/XHO 1 5' fragment of rat kidney PVCR cDNA (Brown, E.M., *et al.*, Nature, 366:575 (1993)), washed at reduced stringency (lX SSC, 0.1% SDS, 50° C.) and exposed for 10 days to autoradiography.

As shown in Figure 3B, the same filter shown in Figure 3A after stripping and hybridization with a ^{32}P-labeled full length 3.8 kb TSC cDNA that was washed at 0.5XSSC, 0.1% SDS at 65° C. and subjected to a 1 hour autoradiogram exposure. Data shown representative of a total of five separate experiments.

These data demonstrate the presence of a 4.4 kb homolog of the mammalian CaR present in poly A+ RNA from urinary bladder together with abundant 3.8 kb thiazidesensitive NaCl contransporter transcript, and suggest no PVCR transcripts are present in other tissues including muscle, heart or testis.

EXAMPLE 3: MOLECULAR CLONING OF SHARK KIDNEY CALCIUM RECEPTOR RELATED PROTEIN (SKCaR-RP)

A shark λZAP cDNA library was manufactured using standard commercially available reagents with cDNA synthesized from poly A+ RNA isolated from shark kidney tissue as described and published in Siner *et al.* Am. J. Physiol. 270:C372-C381, 1996. The shark cDNA library was plated and resulting phage plaques screened using a ^{32}plabeled full length rat kidney CaR (RaKCaR) cDNA probe under intermediate stringency conditions (0.5X SSC, 0.1% SDS, 50°C.). Individual positive plaques were identified by autoradiography, isolated and rescued using

phagemid infections to transfer cDNA to KS Bluescript vector. The complete nucleotide sequence, Figures 4A-E, (SEQ ID NO: 1) of the 4.1 kb shark kidney PVCR related protein (SKCaR-RP) clone was obtained using commercially available automated sequencing service that performs nucleotide sequencing using the dideoxy chain termination technique. The deduced amino acid sequence (SEQ ID NO: 2) is shown in Figures 5A-E. Northern analyses were performed as described in Siner et. al. Am. J. Physiol. 270:C372-C381, 1996. The SKCAR-RP nucleotide sequence was compared to others CaRs using commercially available nucleotide and protein database services including GENBANK and SWISS PIR.

Polymerase chain reaction (PCR) amplification of selected cDNA sequences synthesized by reverse transcriptase (RT) were performed using a commercially available RT-PCR kit from Promega Biotech, Madison, WI. Selective amplification of a conserved region of CaRs (nts 597-981 of RaKCaR cDNA) results in 384 nt cDNA, as shown in Figure 7. This amplified 384 bp was then ligated into the TA cloning vector (Promega Biotech,-Madison, WI) that was then transformed into competent DH5a E. coli cells using standard techniques. After purification of plasmid DNA using standard techniques the 384 nt cDNA was sequenced as described above.

EXAMPLE 4: PVCR EXPRESSION IN TISSUES OF FUNDULUS HETEROCLITUS

To determine if PVCR expression was modulated by adaptation of *Fundulus* to either fresh or salt water, killifish collected in an estuary were first fresh or salt water adapted for an interval of 18 days (chronic adaptation). Selected individuals from each group were then adapted to the corresponding salinity (fresh to salt; salt to fresh) for an interval of 7 days (acute adaptation).

Results are shown in Figure 8. A blot containing RNA (40 ug/lane) prepared from control *Xenopus* kidney (lane 1) or Fundulus heart (containing ultimobranchial tissue) (lanes 2, 5), kidney (lanes 3, 6) and gill (lanes 4, 7) was probed with a 32p-labeled Xenopus PVCR cDNA, washed (.01 x SSC, 650C) and autoradiographed.

As shown in Figure 8, as compared to control MRNA, (lane 1) steady state levels of PVCR MRNA are larger in tissues from seawater adapted fish (lanes 5-7) versus those in fresh water (lanes 2-4).

Fundulus fish were either chronically (Figures 9A and 9B) or acutely (Figures 9C and 9D) adapted to salt water (Figures 9A and 9C) or fresh water (Figures 9B and 9D). The presence of PVCR in kidney tubules was determined by immunocytochemistry. Chronic adaptation to salt water (9A) resulted in increased PVCR expression in kidney tubules as compared to that present in fresh (9B). Kidney tubule PVCR expression in salt water fish was diminished by acute adaptation to fresh water (9C). In contrast, kidney tubule PVCR expression in fresh water fish was increased after acute adaptation to salt water (9D).

EXAMPLE 5: ASSAY FOR PVCR AGONISTS AND ANTAGONISTS USING THE FLOUNDER URINARY BLADDER

To provide further evidence linking Aquatic PVCRs to fish osmoregulation, isolated urinary bladder of winter founder was used to investigate whether PVCRs modulate epithelial cell ion transport. Previous work has demonstrated that the flounder urinary bladder is important in osmoregulation since it allows recovery of both NaCl and water via a thiazide-sensitive NaCl contransport process that has been first generated by the kidney proximal tubule. Water reabsorption from the urine stored in urinary bladder allows for the concentrations of both Mg^{2+} and Ca^{2+} to increase to values as high as 84 mM and 7 mM respectively in marine founders (Elger, E.B., *et al.*, J. Como. Physiol., B157:21 (1987)). 5 Net apical to basolateral water flux (Jv) was measured gravimetrically in 10 minute intervals using individual urinary bladder excised from winter flounder. Briefly, isolated bladders were suspended in a liquid solution (typically a physiologically compatible solution) as described in (Renfro, L.J. Am. J. Physiol. 228:52-61, 1975) the teachings of which are hereby incorporated by reference. The weight of the bladder was measured before and after the experimental period, wherein the experimental period comprised the

period of time that the isolated bladder was exposed to test compound. The compound to be tested (e.g., test compound) was added to both serosal and mucosal solutions. The bladders were dried and weighted as described in Renfro *et al.* The difference in bladder weight prior to and after exposure to test compound is an indication of water reabsorption by the bladder.

Quantification of water reabsorption (Jv) by isolated bladders using the method of Renfro *et al.* showed that Jv was significantly ($p<0.05$) inhibited by addition of 100 AM hydrochlorothiazide ($86\pm2\%$) consistent with the role of the thiazide sensitive NaCl contransporter in this process. Urinary bladder iv was also significantly inhibited by PVCR agonists including 100 μM Gd^{3+} ($75\pm5\%$) and 200 μM neomycin ($52\pm4\%$). (Control Jv values (130 ± 28 μl/gm/hr.) were obtained from animals in September-October and are approximately 21% of the Jv reported by Renfro *et al.* These differences likely reflect seasonal variations in urinary bladder transport.) The half maximal inhibitory concentration for urinary bladder Jv (IC_{50}) for Gd^{3+} (15 μM) was similar to that reported for mammalian CaRs, while the IC_{50} for neomycin (150 μM) was approximately 3 times larger as compared to mammalian CaRs (50 μM). This inhibitory effect of PVCR agonists on Jv was fully reversible. Activation of apical PVCRS by high concentrations of Mg^{2+} and Ca^{2+} resulting from NaCl-mediated water reabsorption from bladder urine would provide for optimal recovery of water by the urinary bladder. This mechanism would permit water reabsorption to proceed until divalent cation concentrations approach levels that promote crystal formation. This overall process is similar to that described for mammalian CaRs in the rat and human IMCD. Additional aspects of these mammalian and teleost renal epithelia may also share other similarities since teleost urinary bladder is both an anatomical and functional homolog of the mammalian mesonephric kidney.

EXAMPLE 6. EXPRESSION/ACTIVATION STUDIES OF SKCaR IN HUMAN EMBRYONIC KIDNEY (HEK) CELLS

The following studies show the following:

1. SKCaR nucleic acid sequence (SEQ ID NO.:1) encodes a functional ion receptor that is sensitive to both Mg2+ and Ca2+ as well as alterations in NaCl.

2. SKCaR's (SEQ ID NO.: 2) sensitivity to Ca2+, Mg2+ and NaCl occur in the range that is found in marine environments and is consistent with SKCaRs role as a salinity sensor.

3. SKCaR's (SEQ ID NO.: 2) sensitivity to Mg2+ is further modulated by Ca2+ such that SKCaR is capable to sensing various combinations of divalent and monovalent cations in seawater and freshwater. These data can be used to design novel electrolyte solutions to maintain fish in salinities different from those present in their natural environment.

SKCaR cDNA (SEQ ID NO.: 1) was ligated into the mammalian expression vector PCDNA II and transfected into HEK cells using standard techniques. The presence of SKCaR protein (SEQ ID NO.: 2) in transfected cells was verified by western blotting. Activation of SKCaR (SEQ ID NO.: 2) by extracellular Ca2+, Mg2+ or NaCl was quantified using a well characterized FURA 2 based assay where increases in intracellular Ca2+ produced by SKCaR activation are detected using methodology published previously the Dr. E. Brown's laboratory (Bai, M., S. Quinn, S. Trvedi, O. Kifor, S.H.S. Pearce, M.R. Pollack, K. Krapcho, S.C. Hebert and E.M. Brown. Expression and characterization of inactivating and activating mutations in the human Ca2+-sensing receptor. J. Biol. Chem., 32:19537-19545 (1996)) and expressed as % normalized intracellular calcium response to receptor activation.

SKCaR (SEQ ID NO.: 2) is a functional extracellular Ca2+ sensor where its sensitivity is modulated by alterations in extracellular NaCl concentrations. As shown

in Figure 10, SKCaR (SEQ ID NO.: 2) is activated by increasing concentrations of extracellular Ca2+ where half maximal activation of SKCaR (SEQ ID NO.: 2) ranges between 1-15 mM depending on the extracellular concentration of NaCl. These are the exact ranges of Ca2+ (1-10 mM present in marine estuarian areas). Note that increasing concentrations of NaCl reduce the sensitivity of SKCaR (SEQ ID NO.: 2) to Ca2+ (see Panel B). This alteration in SKCaR (SEQ ID NO.: 2) sensitivity to Ca2+ was not observed after addition of an amount of sucrose sufficient to alter the osmolality of the extracellular medium. This control experiment shows it is not alterations in cell osmolality effecting the changes observed.

The half maximal activation (EC50) by Ca2+ for SKCaR (SEQ ID NO.: 2) is reduced in increased concentrations of extracellular NaCl. See Figure 11. The EC50 for data shown on Figure 10 is displayed as a function of increasing extracellular NaCl concentrations. Note the EC50 for Ca2+ increases from less than 5 mM to approximately 18 mM as extracellular NaCl concentrations increase from 50 mM to 550 mM.

SKCaR (SEQ ID NO.: 2) is a functional extracellular Mg2+ sensor where its sensitivity is modulated by alterations in extracellular NaCl concentrations. As shown in Figure 12, SKCaR (SEQ ID NO.: 2) is activated in the range of 5-40 mM extracellular Mg2+ and is modulated in a manner similar to that shown in Figures 10 and 11 by increasing concentrations of extracellular NaCl. Similarly, this alteration in SKCaR (SEQ ID NO.: 2) sensitivity to Ca2+ was not observed after addition of an amount of sucrose sufficient to alter the osmolality of the extracellular medium.

The half maximal activation (EC50) by Mg2+ for SKCaR (SEQ ID NO.: 2) is reduced in increased concentrations of extracellular NaCl. See Figure 13. The EC50 for data shown on Figure 12 is displayed as a function of increasing extracellular NaCl concentrations. Note the EC50 for Mg2+ increases from less than 20 mM to approximately 80 mM as extracellular NaCl concentrations increase from 50mM to 550mM.

Addition of 3mM Ca2+ alters the sensitivity of SKCaR (SEQ ID NO.: 2) to Mg2+ and NaCl. See Figure 14. The EC50 for Mg2+ of SKCaR (SEQ ID NO.: 2) is modulated by increasing concentrations of NaCl as shown both in this Figure 14 and in Figure 13. Addition of 3mM Ca2+ to the extracellular solution alters the sensitivity characteristics of SKCaR (SEQ ID NO.: 2) as shown. Note the 3mM Ca2+ increases the sensitivity of SKCaR (SEQ ID NO.: 2) to Mg2+ as a function of extracellular NaCl concentrations.

EXAMPLE 7: DEMONSTRATION OF THE PRESENCE OF A FUNCTIONAL PVCR IN URINARY BLADDER OF WINTER FLOUNDER.

Quantification of water reabsorption (J_v) in isolated bladders (ref. Renfro, J.L. Water and ion transport by the urinary bladder of the teleost Pseudopleuronectes americanus. *Am. J. Physiol.* 228:52-61 (1975) showed that control Jv (130±28 µl/gm/hr; n=14) was significantly ($p<0.05$) inhibited (86±2%) by addition of 100 µM hydrochlorothiazide (18±7 µl/gm/hr; n=6) consistent with the role of the thiazide-sensitive NaCl cotransporter in this process. Urinary bladder J_v was also inhibited significantly by CaR agonists including 100 µM Gd^{3+} (75±5% inhibition; 32±18 µl/gm/hr; n=5) and 200 µM neomycin (52±4% inhibition; 63±10 µl/gm/hr; n=5). The half maximal inhibitory concentration for urinary bladder J_v (IC_{50}) for Gd^{3+} (15±3 µM; n=6) was similar to that reported for mammalian CaRs (See Brown, E.M., G. Gamba, D. Riccardi, D. Lombardi, R. Butters, O. Kifor, A. Sun, M. Hediger, J. Lytton and S.C. Hebert. Cloning and characterization of an extracellular Ca^{2+} sensing receptor from bovine parathyroid. *Nature* 366:575-580 (1993) while the neomycin IC_{50} (150±24 µM; n=6) was approximately 2-3 fold higher than for mammalian CaRs (60-70 µM) (Brown, E.M., G.E.-H. Fuleihan, C.J. Chen and O. Kifor. A comparison of the effects of divalent and trivalent cations on parathyroid hormone release) 3'5'-cyclic-adenosine monophosphate accumulation and the levels of inositol phosphates in bovine parathyroid cells. *Endocrinol.* 127:1064-1071 (1990).

The maximal inhibitory effect for both CaR agonists on J_v was fully reversible as shown in Figure 15.

Response of a single isolated urinary bladder of winter flounder after exposure of its apical membrane to various CaR agonists and hydrochlorothiazide is shown in Figure 15. Water transport (Jv) was measured in a single isolated urinary bladder after sequential exposures to 300 μM Gd3+, 100 mM thiazide and 100 mM Mg2+. Note that full recovery of water transport occurred after exposure to each of these agents. This data validates of the use of isolated urinary bladder as a screening assay.

EXAMPLE 8: IMMUNOCHEMISTRY SHOWING THAT PVCR EXISTS IN OLFACTORY ORGANS

Additional immunocytochemistry experiments were performed using antibody 1169 (the antibody raised against the 23-mer peptide described herein) to localize SKCaR protein where it is present on the apical membrane of the lamellae of the olfactory organ epithelia of the dogfish shark (*Squalus ancanthias*). These data suggest that elasmobranchs possess the ability to "smell" salinity gradients in the marine environments. Furthermore, from this location SKCaR may interact with other odorant receptors that are also 7 transmembrane GTP binding protein receptors.

Figure 31A shows the immunocytochemistry of the lamellae of the olfactory organ epithelia of the dogfish shark (*Squalus ancanthias*) using antisera 1169. Note the brown reaction product indicating specific 1169 antibody binding to the apical membrane of olfactory organ epithelial cells. Figure 31B also is a photograph that shows lamellae that is not subject to antisera 1169, the control.

EXAMPLE 9: PVCRs ISOLATED IN VARIOUS AQUATIC SPECIES

The PVCR has been isolated in several species including winter flounder (sole), summer flounder (fluke) and lumpfish (source of caviar). The PVCR has also been isolated in swordfish and lamprey. In addition, 2 sequences distinct from

SKCaR-I have been obtained from shark indicating there are multiple polyvalent cation sensing receptors in a single species of fish.

Sequences of mammalian CaRs together with the nucleotide sequence of SKCaR (SEQ. ID NO.: 1 and SEQ ID NO: 2) were used to design degenerate oligonucleotide primers to highly conserved regions in the extracellular domain of polyvalent cation receptor proteins using standard methodologies (See GM Preston, Polymerase chain reaction with degenerate oligonucleotide primers to clone gene family members, Methods in Mol. Biol. Vol. 58 Edited by A. Harwood, Humana Press, pages 303-312, 1993). Using these primers, cDNA or genomic DNA from various fish species representing important commercial products are amplified using standard PCR methodology. Amplified bands are then purified by agarose gel electrophoresis and ligated into appropriate plasmid vector that is transformed into a bacterial strain. After growth in liquid media, vectors and inserts are purified using standard techniques, analyzed by restriction enzyme analysis and sequenced where appropriate. Using this methodology, a total of 5 nucleotide sequences from 4 fish species were amplified.

Two additional nucleotide sequences were isolated from the Dogfish shark (*Squalus ancanthias*),same species as SKCaR-I (SEQ ID NO:2). Two nucleotide sequences, SEQ ID NO: 3 (Figurea 16A-B) and SEQ ID NO: 5 (Figure 19), were isolated from genomic SEQ ID NO: 3 or cDNA obtained from shark rectal gland (SEQ ID NO: 5). Both SEQ ID NOs: 3 and 5 are unique as compared to corresponding regions of the nucleotide sequence of SKCaR-I (SEQ ID NO:1). SEQ ID NOs: 4 and 6 (Figures 17 and 20, respectively) represent the corresponding amino acids of putative open reading frames of SEQ ID NOs: 3 and 5. Thus, these 2 sequences represent at least 1 (different fragments of a single other gene) or possibly 2 calcium polyvalent cation sensing receptor proteins distinct from the SKCaR-I. Figures 18 and Figures 21 show the nucleotide sequences for SEQ ID NOs: 3 and 5, respectively, and the corresponding deduced amino acid sequences (SEQ ID NOs: 4 and 6, respectively).

SEQ ID NO: 3 is composed of 784 nucleotides (nt) containing an open reading frame coding for 261 amino acids. SEQ ID NO: 3 is similar, but not identical to the corresponding sequence in the extracellular domain of SKCaR I (SEQ ID NOs: 1 and 2) from nt. 1087-1836.

SEQ ID NO: 4 is composed of 261 Amino acids corresponding to the putative open reading for SEQ ID NO: 3.

SEQ ID NO: 5 is composed of 598 nucleotides (nt) containing an open reading frame coding for 198 amino acids and was obtained using oligonucleotide primers different from those used for SEQ ID NO: 3. SEQ ID NO: 5 is similar, but not identical to the corresponding sequence in the extracellular domain of SKCaR I (SEQ ID NOs: 1 and 2) from nt. 2279-2934.

SEQ ID NO: 6 comprises 198 Amino acids corresponding to the putative open reading for SEQ ID NO: 4.

Winter Flounder (*Pleuronectes americanus*) marine flatfish species was also isolated using the techniques described herein. SEQ ID NO: 7 was obtained from cDNA prepared from urinary bladder where functional data show presence of PVCR protein. SEQ ID NO: 8 corresponds to amino acids in the putative open reading frame of SEQ ID NO: 7.

SEQ ID NO: 7 is composed of 594 nucleotides (nt) containing an open reading frame coding for 197 amino acids. SEQ ID NO: 7 is homologous to the corresponding sequence in the extracellular domain of SKCaR I (SEQ ID NOs: 1 and 2) from nt. 2279-2937.

SEQ ID NO: 8 comprises the 197 Amino acids corresponding to the putative open reading frame of SEQ ID NO: 7.

Summer Flounder (*Paralichthus dentalus*) is another marine flatfish species that was isolated using methods, as described herein. SEQ ID NO: 9 was obtained from cDNA prepared from urinary bladder that is similar in function to the urinary bladder of winter flounder. SEQ ID NO: 10 contains amino acid corresponding to the putative open reading frame of SEQ ID NO: 9.

SEQ ID NO: 9 is composed of 475 nucleotides (nt) containing an open reading frame coding for 157 amino acids. SEQ ID NO: 9 is homologous to the corresponding sequence in the extracellular domain of SKCaR I (SEQ ID NOs: 1 and 2) from nt. 2279-2934.

SEQ ID NO: 10 has 157 Amino acids corresponding to the open reading frame for SEQ ID NO: 9.

Lumpfish (*Cyclopterus lumpus*) is an arctic marine fish that was isolated. Lumpfish is the sole source of lumpfish caviar. SEQ ID NO: 11 was obtained from cDNA prepared from the urinary bladder of lumpfish. SEQ ID NO: 12 is the corresponding amino acid sequence of the putative 435 amino acid open reading frame of SEQ ID NO: 11.

SEQ ID NO: 11 is composed of 1308 nts. that are homologous to the corresponding sequence in the extracellular domain of SKCaR I (SEQ ID NO: 1 and 2) from nt 1087-2441.

SEQ ID NO: 12 comprises the 435 Amino acids corresponding to the putative open reading frame for SEQ ID NO: 11.

Primer sequences for PCR of PVCR clones:		Sequences derived from the following SEQ ID NOs:
dSK-F1	5'-GCI GCT GAY GAY GAY TAY GG-3'(SEQ ID NO.: 15)	3, 11
dSK-R2	5'-CCA IGC YTC IAG YTT YTT DAT RTC-3"(SEQ ID NO.: 16)	3
dSK-F3	5'-TGT CKT GGA CGG AGC CCT TYG GRA TCG C-3'(SEQ ID NO.: 17)	5, 7,9
dSK-R3	5'-ATA GGC KGG RAT GAA RGA KAT CCA RAC RAT GAA G-3'(SEQ ID NO.: 18)	7
dSK-R4	5'-GGC KGG RAT GAA RGA KAT CCA RAC RAT GAA G-3'(SEQ ID NO.: 19)	5, 9, 11

I=deoxyinosine, N=A+C+T+G, R=A+G, Y=C+T, M=A+C, K=T+G, S=C+G, W=A+T, H=A+T+C, B=T+C+G, D=A+T+G, V=A+C+G

EXAMPLE 10: ALTERING THE BODY COMPOSITION OF FISH AND DEFINING SALINITY LIMITS

Winter and Summer Flounder can be grown and maintained in recycling water systems. Groups of both winter (*Pleuronectes americanus*) and summer (*Paralichthus dentalus*) flounder were maintained in multiple modular recycling water system units that are composed of a single 1 meter fish tank maintained by a 1 meter biofilter tank located directly above it. The upper tank of each unit contains 168 sq. ft. of biofilter surface area that will support a maximum of 31 lbs of flounder, while maintaining optimal water purity and oxygenation conditions. Each unit is equipped with its own pump and temperature regulator apparatus. Both the temperature and photoperiod of each unit can be independently regulated using black plastic curtains that partition each tank off from its neighbor. The inventors have a total of 12 independent modular units that permit 3 experiments each with 4 variables to be performed simultaneously. Using this experimental system, the following data have been obtained.

Salinity survival limits for winter and summer flounder with a constant ratio of divalent and monovalent ions were determined. The survival limit of both winter and summer flounder in waters of salinities greater than normal seawater (10 mM Ca2+, 50 mM Mg2+ and 450 mM NaCl) is water containing twice (20 mM Ca2+, 50 mM Mg2+ and 900 mM NaCl) the normal concentrations of ions present in normal seawater. In contrast, the survival limit of both winter and summer flounder in waters of salinity less than normal seawater is 10% seawater (1 mM Ca2+, 5 mM Mg2+ and 45 mM NaCl).

Flounder grown and/or maintained in low and hypersalinities possess different fat contents and taste as compared to flounder maintained in normal sea water. Use of a fully recycling water system permits growth of flounder at vastly different salinities. Groups of flounder (n=10) were adapted over a 15 day interval and maintained at either low salinity (LS) (e.g., at 10% normal seawater), normal seawater (NS) or hypersalinity (HS) (e.g., 2X seawater) for intervals of 3 months, under otherwise

identical conditions. Survival among the 3 groups were comparable (all greater than 80%) and there were no differences in the electrolyte content of their respective sera. Analyses of fillet muscle from summer flounder for total fat, protein and moisture content are shown on Table I.

TABLE I: Comparison of Total Fat, Protein and Moisture Content of Muscle from Flounders Grown at Differing Water Salinities for 3 months. All values an average of 4 individual fish.

Salinity	10% Seawater	Normal Seawater	2X Seawater
% Total Fat	3.36±0.43*	2.59±0.31*	1.98±0.66*
% Total Protein	19.6±0.23	19.9±0.42	18.99±0.34
% Moisture	74.7±2.1	75.1±1.8	73.8±2.5

*Values significantly different from each other ($p<0.05$).

Muscle from low salinity flounder contains approximately 30% higher fat content as compared to flounder maintained in normal seawater and approximately 70% greater fat content when compared to flounder maintained in 2X seawater (e.g., the fat of a flounder maintained in normal salinity is 40% greater than flounder maintained in twice seawater). These differences appear selective because no significant differences were observed in either muscle protein or moisture content.

Furthermore, fillets were sampled in a blinded protocol where tasters (n=6) were offered either raw or cooked fillets without knowledge of salinity conditions. Tasters could distinguish little difference between the taste of fillets of individual fish from each specific salinity group. However, when asked to compare fillets from flounder grown at differing salinities, a majority (5/6) clearly distinguished a taste difference between fillets from fish maintained at 10% salinity describing them as "sweet and buttery tasting with a soft consistency" as compared to fillets from fish maintained at either normal seawater or 2X seawater that were described as "wild and fishy tasting with a firmer consistency. These data provides evidence that "finishing" growth of winter

flounder at different water salinities can be used to alter the taste and fat content of the resulting fillets in summer and winter flounder.

Groups of tagged hatchery raised summer flounder obtained from identical broodstock were exposed to either 10% seawater or 2X seawater for an interval of 3 months under conditions identical to that described above. There were no significant differences in either length or width in fish maintained 10% seawater or 2X seawater. However, there was a significant difference in the weights of the respective fish where 10% seawater fish weighted $80\pm14\%$ (n=10) more than summer flounder maintained in 2X seawater. Moreover, the summer flounder maintained in 10% seawater were nearly twice (2.1 ± 0.4 times n=6) as thick as compared to fish maintained in 2X seawater. These data show that flounder maintained at different water salinities exhibit significant differences in the thickness of their fillets. Thus, flounder could be “finished” using water of differing compositions to alter the thickness of their fillets.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.